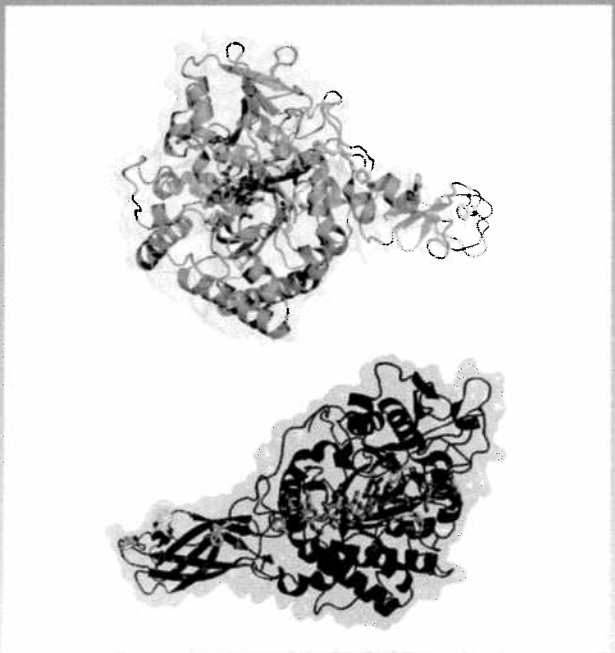
Biotechnology & Genetic Engineering Reviews

Volume 27



Edited by

Stephen E. Harding

Nottingham
University Press

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2010		**00-00-2010 to 00-00-2010**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Biotechnology & Genetic Engineering Reviews, Vol 27	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Air Force Research Laboratory,AFRL/RXXL, Microbiology and Applied Biochemistry,Tyndall Air Force Base,FL,32403**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
Biotechnology and Genetic Engineering Reviews, Vol 27, 95-114 (2010)

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**14**	

Biotechnology and Genetic Engineering Reviews - Vol. 27, 95-114 (2010)

Co-immobilized coupled enzyme systems in biotechnology

LORENA BETANCOR[1*] AND HEATHER R. LUCKARIFT[2,3*]

[1]Madrid Institute for Advanced Studies, Campus Universitario de Cantoblanco, c/ Einstein, 13 Pabellon C 1°Planta, E-28049, Madrid, Spain. [2]Air Force Research Laboratory, AFRL/RXQL, Microbiology and Applied Biochemistry, Tyndall Air Force Base, Florida 32403. [3]Universal Technology Corporation, 1270 N. Fairfield Road, Dayton, Ohio, 45432, USA

Abstract

The development of coimmobilized multi-enzymatic systems is increasingly driven by economic and environmental constraints that provide an impetus to develop alternatives to conventional multistep synthetic methods. As in nature, enzyme-based systems work cooperatively to direct the formation of desired products within the defined compartmentalization of a cell. In an attempt to mimic biology, coimmobilization is intended to immobilize a number of sequential or cooperating biocatalysts on the same support to impart stability and enhance reaction kinetics by optimizing catalytic turnover.

There are three primary reasons for the utilization of coimmobilized enzymes: to enhance the efficiency of one of the enzymes by the *in-situ* generation of its substrate, to simplify a process that is conventionally carried out in several steps and/or to eliminate undesired by-products of an enzymatic reaction. As such, coimmobilization provides benefits that span numerous biotechnological applications, from biosensing of molecules to cofactor recycling and to combination of multiple biocatalysts for the synthesis of valuable products.

*To whom correspondence may be addressed (lorena.betancor@uam.es or heather.luckarift.ctr@tyndall.af.mil)

Abbreviations: ATP- adenosine triphosphate; EDTA- ethylenediaminetetraacetic acid; CALB- Candida antarctica B lipase; GOX- glucose oxidase; HRP- horse radish peroxidase; ABTS- 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); NADPH- Nicotinamide adenine dinucleotide phosphate reduced form; NADH- Nicotinamide adenine dinucleotide reduced form; CoA- Coenzyme A; FAD- flavin adenine dinucleotide; FADH2- flavin adenine dinucleotide reduced form; GTP- Guanosine-5'-triphosphate; GDP- Guanosine-5'-diphosphate; DNA- Deoxyribonucleic acid

Introduction

The best example of coimmobilized enzymes collaborating *in situ* is in living cells. Enzymes constitute the basis of metabolism in all living beings and, in a perfectly concerted succession of catalytic steps, form a network of reactions that make life possible. Enzymes that function to form a complete process such as metabolic cycling are physically associated so as to ensure substrate channeling without diffusion limitations. This metabolic organization is intrinsic to cells and although whole cells may be visualized as a random mix of enzymes, recent studies suggest a specific intracellular organization of enzymes specifically to allow for channeling of enzyme intermediates (Beeckmans *et al.*, 1993; Huang *et al.*, 2001). Many of the enzymes of the citric acid cycle, for example, are inhibited by their reactants, products, intermediates or even cofactors involved in the cycle; functioning as a continuous unit in which inhibitory intermediates are immediately removed, maintains enzyme functionality throughout the cycle.

In an attempt to mimic nature, science has benefited from the high efficiency, selectivity and specificity of coupled enzyme systems for detection, diagnosis or synthesis of industrially relevant molecules (Schoevaart and Kieboom, 2001; Bruggink *et al.*, 2003). One primary advantage of using multi-enzyme systems in biocatalysis is the ability to convert a starting material into a desired product without the need to separate or isolate intermediate products. Moreover, some intermediates may not be available or stable if added *ex situ*. In this instance, coimmobilization of enzymes can make a biocatalytic pathway more efficient by limiting the diffusion of unstable intermediates into the surrounding media. Biocatalysis can also offer a significant advantage over chemical catalysis by eliminating the defined reaction environments required in organic synthesis. The modification of penicillin to 6-aminopenicillanic acid, for example, is now predominantly manufactured by an enzymatic process using immobilized penicillin acylases in a single-step biocatalytic reaction that replaces the traditional three-step chemical reaction and eliminates the need for harsh solvents and the cost of operating the reaction at extreme temperatures (-40°C) (*Figure 1*) (Averill *et al.*, 1999; Zaks, 2001[1]).

Herein, we will review what we consider the most current relevant examples of coupled-enzyme systems used for biosensing or biocatalytic purposes. The immobilization strategies will be described with special emphasis on those that have improved the process in which they were applied and that could potentially be used with other enzyme species. Particular attention will be given to new nanoscale architectures that are increasingly emerging as interesting supports for nanoscale biotechnological applications. This review is not intended as an exhaustive summary of all coimmobilized or cascade enzyme systems but rather aims to demonstrate the breadth of innovative immobilization strategies, the diverse range of applications that may benefit from immobilized biocatalysis and highlight pertinent examples that exemplify the technology.

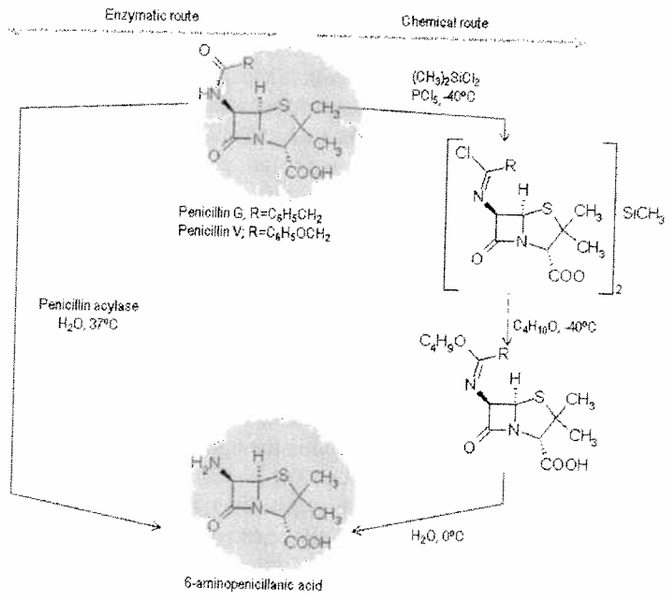


Figure 1. Enzymatic and chemical routes to 6-aminopenicillanic acid from penicillin G or V.

Fundamentals of immobilized enzymes

Inherently, the soluble nature of enzymes presents specific restrictions for biotechnological applications: specifically poor stability, difficulty of separation, product contamination and limited reuse. Enzyme immobilization is therefore commonly used to overcome the limitations of enzyme utilization, as stabilization is often provided against heat, organic solvents and/or changes in pH (Grazú *et al.*, 2005; Irazoqui *et al.*, 2007; Mateo *et al.*, 2007b; Montes *et al.*, 2007; Kim *et al.*, 2008; Hanefeld *et al.*, 2009). Furthermore, immobilized enzymes can be reused, often through many cycles, thereby minimizing costs and time of analysis and in certain applications facilitating the continuous use of the biocatalyst (Berne *et al.*, 2006; Filice *et al.*, 2009). Stabilization may also fortuitously improve enzyme properties by locking the protein structure in a configuration that enhances substrate specificity and reduces the effect of inhibitors (Mateo *et al.*, 2007b; Sheldon, 2007).

A wide variety of techniques are now available for enzyme attachment to a variety of supports (Girelli and Mattei, 2005; Mateo *et al.*, 2007b; Betancor and Luckarift, 2008). Immobilization techniques generally include chemical or physical mechanisms. Chemical immobilization methods mainly include enzyme attachment to the matrix by covalent bonds or other interactions and cross-linking between the enzyme and the matrix. Physical methods involve the entrapment of the enzymes within an insoluble matrix. A combination of chemical and physical methods has facilitated in certain circumstances for the immobilization of different enzyme species in the same composite (Kreft *et al.*, 2007). The requirements of different enzymes are inherently

varied and specific conditions are often needed for a defined application. Unfortunately, there is at the present time, no generic method for enzyme stabilization that will be optimal for all enzyme systems, but a toolbox of versatile methodologies is now well documented in the literature and provides examples in which cooperating enzymes have been immobilized for various applications (Nahalka *et al.*, 2003; Berne *et al.*, 2006; Salinas–Castillo *et al.*, 2008).

Considerations for coimmobilization of enzymes

The limitations of biocatalysis are particularly evident when attempting to utilize a multitude of enzyme activities in concert. Coimmobilization of cooperating enzymes requires specific optimization to balance the catalytic components and therefore necessitates screening of suitable immobilization methods, design and preparation of the appropriate immobilization carriers, and analysis of the relevant reaction kinetics and mass transfer characteristics to determine the optimum reaction conditions (El–Zahab *et al.*, 2004; Lopez–Gallego *et al.*, 2005; Sun *et al.*, 2009). Immobilization of more than one enzyme on the same support, however, is especially challenging as it needs to preserve the catalytic activity of all the enzymes involved in the system and ideally improve stability. An ideal immobilization design should confer an overall operational stabilization to each of the enzymes involved; otherwise the half-life of the composite will be limited by the most unstable catalytic component.

The beauty of combined biological enzymes over purely chemical cascades is that enzymes inherently function in the same physiological environments, i.e., aqueous solvents, moderate temperature and defined pH. An elegant example of pH control of enzymes was utilized in the four-step enzymatic conversion of glycerol into a heptose in which pH switching was used to temporarily control the on/off catalysis of enzymes involved within the cascade (Schoevaart *et al.*, 2001).

Immobilization of sequentially acting enzymes within a confined space increases the catalytic efficiency of conversion due to a dramatic reduction in the diffusion time of the substrate. Moreover, the *in-situ* formation of substrates generates high local concentrations that lead to kinetic enhancements and can equate to substantial cost savings (Van De Velde *et al.*, 2000; El–Zahab *et al.*, 2004). The interest in reducing diffusion limitations and maximizing the functional surface area to increase enzyme loading has prompted the emergence of new nanoscaffolds that could potentially support enzyme immobilization (Kim *et al.*, 2008). Among them, the bioinspired formation of silica nanoparticles provides a versatile new technology for enzyme immobilization with several inherent advantages: inexpensive, rapid, mild, robust and stabilizing for the entrapped enzymes (Betancor and Luckarift, 2008; Luckarift, 2008; Vamvakaki *et al.*, 2008). This particular immobilization support has been used to couple sequentially acting enzymes with very good results (Luckarift *et al.*, 2007). The application utilized individual enzymes encapsulated in silica, packed into microfluidic chips and then connected in series to allow the flow of reaction products from one step to the next for the synthesis of a metabolite relevant to antibiotic synthesis. This type of sequential processing has numerous applications in catalysis including the ability to change the direction and ordering of the reaction sequence (Lee *et al.*, 2003; Ku *et al.*, 2006; Logan *et al.*, 2007). Logan *et al.*, demonstrated an elegant example of spatially separated multi-enzyme reactions by patterning enzymes onto monoliths using covalent attachment. Glucose oxidase and horseradish peroxidase were immobilized sequentially in a flow-through system whereby glucose oxidase converts glucose to gluconolactone, liberating hydrogen peroxide, which is subsequently utilized by horseradish peroxidase to oxidize amplex red to the red fluorescent product, resorufin. Interestingly, red fluorescence was observed only when glucose and amplex red were flushed in a forward direction. Reversing the flow essentially eliminated fluorescence and confirmed the correct sequential ordering of the catalytic steps. In an additional step, invertase was added prior to the reaction scheme to allow the *in-situ* hydrolysis of sucrose to glucose (and fructose) and extend the substrate range of the reaction system (Logan *et al.*, 2007).

Coimmobilization of enzymes in biosensing

Generally, coimmobilized enzymes serve one of two primary purposes: to channel an intermediate reaction product directly to a secondary enzyme and reduce the loss of intermediates as a result of instability or diffusion. Alternatively, a coimmobilized enzyme may be required to recycle a cofactor to maintain catalytic turnover and eliminate the need to continually add cofactor to the reaction (*Figure 2*).

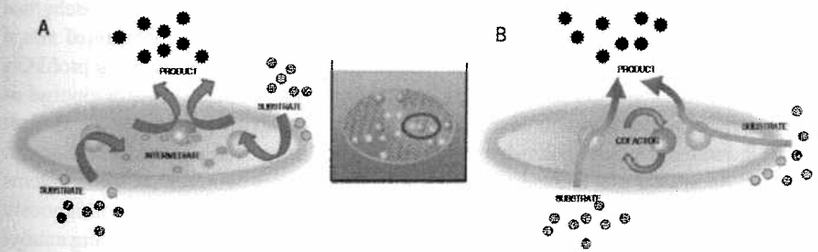


Figure 2. Coimmobilized enzyme systems for *in-situ* generation of an intermediate (A) or recycling of a cofactor (B).

In sensing applications, the use of more than one enzyme species often allows the sensitivity of the analytical method to be increased, expanding the range of applications for detection of numerous substrates at otherwise undetectable concentrations (Salinas–Castillo *et al.*, 2008). Numerous bi-enzyme systems have been reported for glucose detection, for example, by combining a secondary enzyme to enhance or improve the sensitivity and selectivity of the signal. Peroxidase and glucose oxidase, were immobilized onto carbon nanotubes using polypyrrole or Nafion® to provide a basis for bi-enzymatic glucose biosensors (Zhu *et al.*, 2007; Jeykumari and Narayanan, 2008). Glucose oxidase and horseradish peroxidase with fluorogenic detection by resorufin is a combination often used in bi-enzyme sensing systems due to the sensitivity of peroxide production coupled with fluorescence, which significantly increases the detection of the initial reaction product (hydrogen peroxide).

Recently, researchers investigated the ability to define the spatial orientation of enzymes by utilizing specific 'capture' oligomers to tag enzymes with a short nucleotide sequence and align the proteins in a specific location on a DNA backbone (Muller

and Niemeyer, 2008). Using this method glucose oxidase and horseradish peroxidase were coimmobilized onto microplates. The catalytic activity was hampered by steric interactions as a result of specific organization of the enzymes within the system, but, when organized to limit steric affects, demonstrated the potential for enhanced detection specificity of glucose. Signal detection was based on direct coupling of the conversion of glucose (and the concomitant production of hydrogen peroxide) with the fluorescence based detection of resorufin. Similarly, the coimmobilized orientation of luciferase and oxidoreductase was used to catalyze flavin mononucleotide reduction and aldehyde oxidation (Niemeyer *et al.*, 2002).

Not all coimmobilization strategies, however, rely on catalytic cooperativity. Wang *et al.*, reported the coimmobilization of glucose oxidase and heparin by electropolymerization into a polymeric film (Wang *et al.*, 2000). The immobilized glucose oxidase provides an amperometric measure of glucose concentration in blood with application to needle-type implantable glucose biosensors. Implantable sensors, however, are susceptible to fouling upon continuous exposure to biological fluids. The inclusion of heparin (as an anticoagulant) extends the biocompatibility and hence reusability of the device.

Silica sol–gels have provided a broad and versatile basis for many examples of immobilized multi-enzyme systems but have limitations associated with drying and cracking. In an alternate design for amperometric detection of glucose, a hybrid silica sol–gel was used to encapsulate glucose oxidase and glucose-6-phosphate dehydrogenase. The silica sol–gel was formed from the hydrolysis of a mixture of silane precursors to create a three-dimensional structure that limited the cracking problems associated with conventional sol–gels (Liu and Sun, 2007). The addition of glucose-6-phosphate provides a competitive catalytic sink that utilizes a stoichiometric amount of ATP and results in a detection method for both glucose and ATP. The addition of ATP to the system is a typical example of one of the limitations of enzyme systems and particularly of multi-enzyme systems in that cofactors have to be continuously added or an additional enzyme included, for the cofactor to be recycled during catalysis. The conversion of glucose into riboflavin, for example, can be performed by six catalytic enzymes working in concert, but an additional two enzymes are required to recycle cofactors during synthesis. The eight-enzyme reaction functions entirely in an aqueous system as a random mixture of enzymes and, as such, the reusability of the enzymes within the system is limited (Romisch *et al.*, 2002).

Coimmobilization of enzymes in biocatalysis

The majority of multi-enzyme cascades in biocatalysis have been developed for carbohydrate synthesis or sugar conversions as enzymatic oligosaccharide synthesis using recombinant glycosyl transferases overcomes many of the hurdles associated with chemical synthesis. Many enzymes of the nucleotide biosynthetic pathway have now been well studied and recombinantly expressed. The attachment of a hexahistidine "tail" to the required biocatalytic enzymes allows for affinity binding to a metal-coated support (Nahalka *et al.*, 2003). By varying the number of enzymes in the coimmobilization step, four-enzyme (Superbeads I) or seven-enzyme cascades (Superbeads II) have been demonstrated that allow for efficient biocatalysis of a versatile range of oligosaccharides, depending upon the starting saccharide units.

Often, coimmobilization of enzymes helps to prevent inactivation that may arise due to high localized concentrations of intermediates or reaction products that may act as inhibitors upon catalytic activity. Limiting the local concentration of hydrogen peroxide, for example, is a common goal to preserve enzyme activity. Coimmobilization of peroxidase and glucose oxidase in a polyurethane foam, for example, resulted in the *in situ* generation of hydrogen peroxide from glucose and glucose oxidase but at a low internal concentration; sufficient to allow the catalytic turnover of the peroxidase enzyme without inactivation that arises from direct addition of peroxide (Van De Velde *et al.*, 2000). Coimmobilization may also provide an added benefit and create an apparent change in the enzymatic activity of a protein, due to reaction synergy. Soybean peroxidase, thus acts as an apparent oxygen transferase when immobilized with glucose oxidase (Van De Velde *et al.*, 2000).

In the conversion of dextran, the enzyme dextransucrase must be protected from dextranase for the two enzymes to work together. As dextran forms it remains associated with the dextransucrase enzyme and can be inactivated by dextranase. Successful coimmobilization of the two enzymes, however, was achieved by preliminary absorption of dextranase onto hydroxyapatite before coimmobilization with dextransucrase in alginate microbeads (Erhardt *et al.*, 2000). This method of compartmentalization is a common theme in coimmobilized systems to spatially separate conflicting catalytic activities. Compartmentalization, for example, was used to separate glucose oxidase and peroxidase in a shell-in-shell microcapsule (Kreft *et al.*, 2007). Polyelectrolyte layers deposited onto calcium carbonate microcapsules can later be dissolved with EDTA to leave an empty shell-in-shell structure with peroxidase in the inner shell and glucose oxidase in the outer shell. Hydrogen peroxide is generated in the outer shell (by the catalytic mechanism of glucose oxidase) and diffuses into the inner compartment, where peroxidase utilizes the peroxide in the conversion of amplex red to resorufin. The red fluorescence of the resulting resorufin can be imaged to demonstrate the architecture of the shell structure and confirm the multistep biocatalytic reaction. Similarly, hemoglobin and glucose oxidase were combined to create microcapsules through a similar layer-by-layer deposition to produce microcapsules that were responsive to the concentration of glucose (Qi *et al.*, 2009).

Initial studies for compartmentalization of enzymes relied on the use of phospholipid liposomes, which mimic the natural cell structure but are difficult to handle due to mechanical fragility; however, vesicles prepared by layer-by-layer techniques as described above have overcome some of the mechanical limitations. There are some examples in the literature that follow strategies for enzyme coimmobilization that resemble cell-like conditions, for instance, to control the order in which enzymes react or to protect one of them from the action of degrading byproducts. van Dongen *et al* have worked on the compartmentalization of sequentially acting enzymes immobilized in the same structure (van Dongen *et al.*, 2009). In an effort to mimic cell-like enzymatic cascades, these researchers developed spherical aggregates called polymersomes to spatially distribute enzymes acting in tandem (*Figure 3*).

There are still specific considerations in this approach, namely that the size of the enzymes may limit expression in certain compartments. The resulting "nanoreactors" contain glucose oxidase (in the lumen), lipase (in the membrane bilayer) and horseradish peroxidase (on the surface) (van Dongen *et al.*, 2009). Glucose oxidase, was included in the polymersome lumen as it was thought that its size might disrupt

the structure if included in the membrane bilayer. Similarly, the hydrophobicity and structural affinity of the enzymes was also considered in respect to positioning. Specifically, azido-functionalization of the surface was required to enable active attachment of horseradish peroxide at the surface. Although the assembly did not provide a catalytic advantage over the use of the soluble enzyme mixture, this strategy can be adapted for future applications with enzyme systems that otherwise could not be coimmobilized.

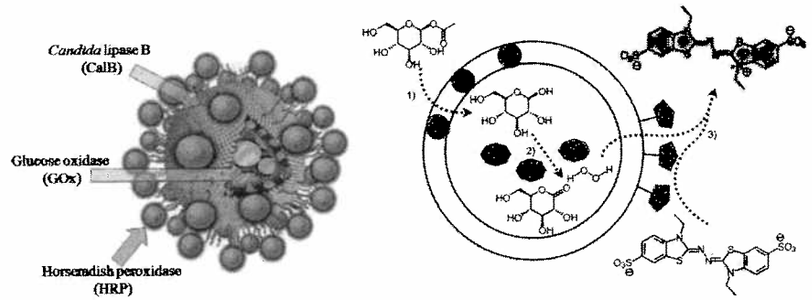


Figure 3. Compartmentalization of enzymes in a polymersome. Acetate-protected glucose is deprotected by *Candida* lipase B (CalB) at the polymersome membrane to give glucose, which is oxidized by glucose oxidase (GOx) in the inner compartment and generates hydrogen peroxide which is used by horseradish peroxidase (HRP) to oxidize ABTS [2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] at the polymersome surface.[van Dongen *et al.*, A three-enzyme cascade reaction through positional assembly of enzymes in a polymersome nanoreactor. Chemistry – A European Journal, 2009, 15, 1101. Copyright Wiley–VCH Verlag GmbH & Co. KGaA, reproduced with permission].

One similar consideration for coimmobilization is in preferential binding, particularly if the method of immobilization relies on covalent attachment. Lipase, trypsin and α-amylase, for example, were bound to fabrics using covalent fixation, but preferential binding was observed for trypsin over α-amylase due to the differing reactivity of the enzymes towards the activated support (Nouaimi-Bachmann *et al.*, 2007). Alternatively, non-sequential encapsulation favors a much more random organization, which may fortuitously favor protein–protein interaction. Nitrobenzene nitroreductase and glucose-6-phosphate dehydrogenase, for example, were coimmobilized by encapsulation in silica spheres that were formed by a polymer-templated silicification reaction (Betancor *et al.*, 2006). Nitrobenzene nitroreductase was used to catalyze the hydroxylation of nitrobenzene, a reaction that requires β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Glucose-6-phosphate dehydrogenase was co-encapsulated as a catalytic sink to allow the continuous conversion of $NADP^+$ to NADPH and provide a continuous supply of NADPH to the system (*Figure 4*). The resulting coimmobilized system was able to convert nitrobenzene at millimolar concentrations continuously (~ 8 hours) into the resulting hydroxylaminobenzene with excellent efficiency (>90%). In the absence of glucose-6-phosphate, conversion of nitrobenzene was minimal as NADPH became rapidly depleted from the system.

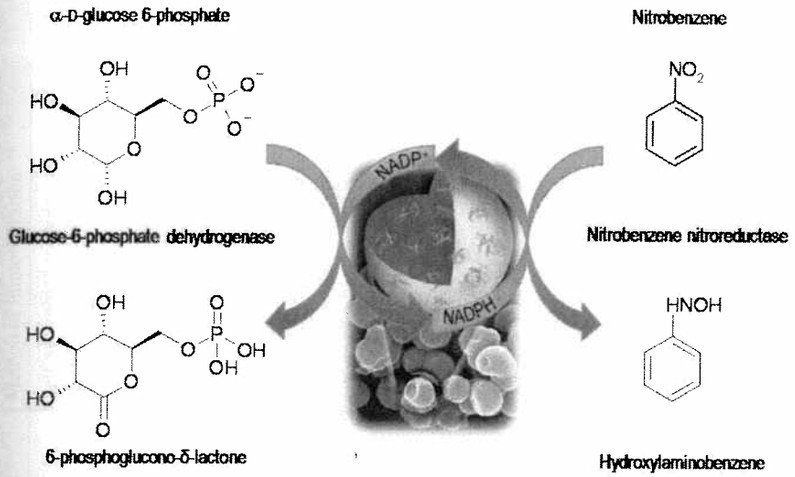


Figure 4. Schematic for the enzymatic hydroxylation of nitrobenzene with cofactor recycling via coimmobilization of enzymes in silica nanospheres.

Application of coimmobilized multi-enzyme systems

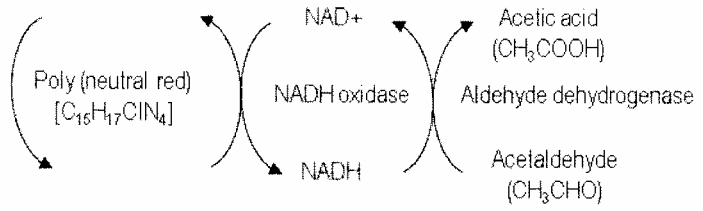
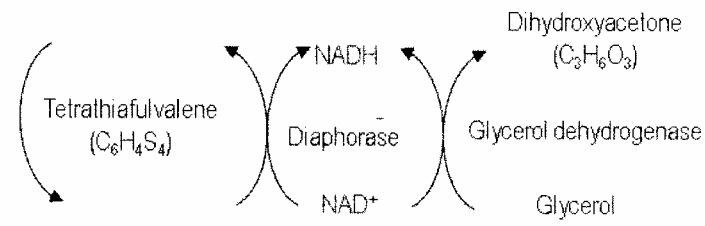
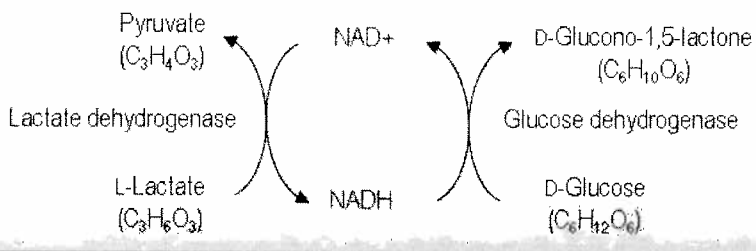
Multi-enzyme combinations now range from simple bi-enzymatic systems to complex multi-enzyme systems that mimic biochemical cycles (Table 1). There are numerous real world applications to these devices such as the determination of glycerol in wines (Gamella *et al.*, 2008). An amperometric biosensor, for example, based on glycerol dehydrogenase and diaphorase in which diaphorase recycles NAD^+ to NADH acts as a bi-enzyme system. An alternate tri-enzyme system uses glycerol kinase, glycerol-3-phosphate oxidase and peroxidase. Both systems cause the reduction and oxidation of tetrathiafulvalene, which causes a redox response that is directly related to the concentration of glycerol. Similarly, determination of acetaldehyde in alcoholic beverages can be monitored by coimmobilization of NADH oxidase with aldehyde dehydrogenase (Ghica *et al.*, 2007). Cross-linking the enzymes with glutaraldehyde, or entrapment in sol–gel, both provide stabilization to the enzyme but with a variation in the sensitivity of measurements dependent upon the immobilization strategy (up to 60 μM for sol–gel encapsulation and 100 μM for glutaraldehyde immobilization). The bi-enzymatic sensors showed improvement over aldehyde dehydrogenase alone with NAD^+ added exogenously to the reaction.

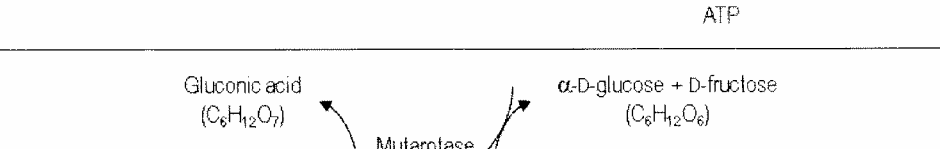
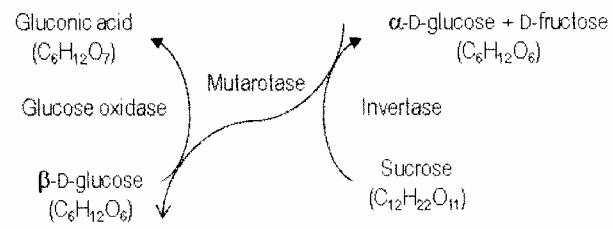
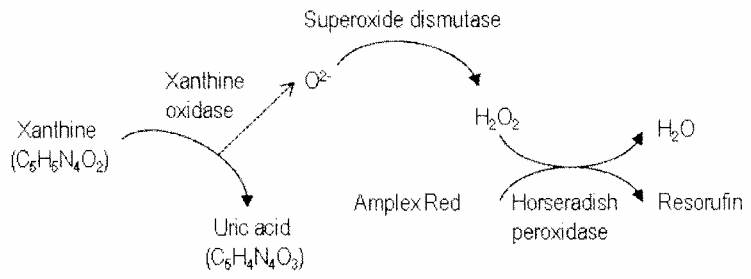
Future directions

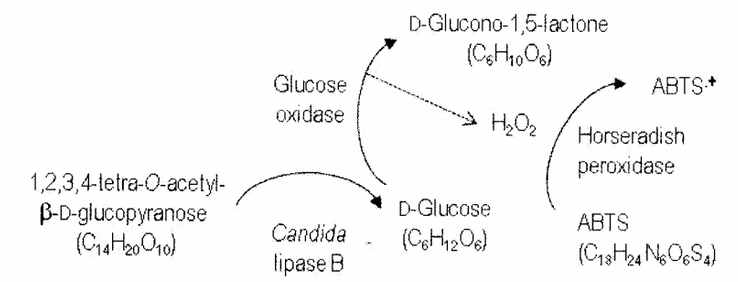
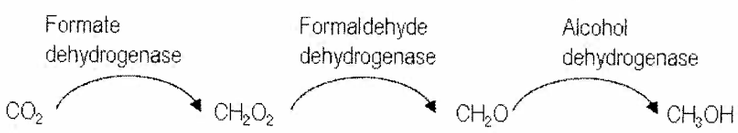
LEARNING FROM CELLS

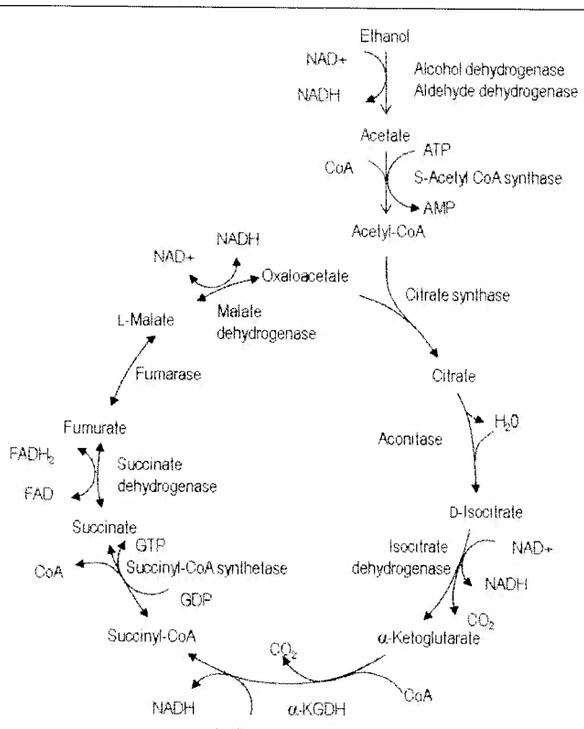
There is a wealth of knowledge that we can learn from cells regarding how enzymes function in a concerted manner; compartmentalization strategies (e.g., differences in

Table 1. Examples of coimmobilized enzymatic reactions

	Reaction	Method of immobilization
Bi-enzyme		Sol–gel encapsulation and glutaraldehyde cross-linking (Ghica *et al.*, 2007)
		Self-assembled monolayers (Gamella *et al.*, 2008)
		Covalent attachment via glutaraldehyde and polyethylene glycol spacers (El–Zahab *et al.*, 2004)

	Reaction	Method of immobilization
Tri-enzyme		Self-assembled monolayers (Gamella *et al.*, 2008)
		Attachment to a natural protein membrane via glutaraldehyde (Majer-Baranyi *et al.*, 2008)
		Sol–gel encapsulation (Salinas–Castillo *et al.*, 2008)

Reaction	Method of immobilization
	Compartmentalized immobilization in polymersomes (van Dongen *et al.*, 2009)
	Encapsulation in protamine-mediated titania particles (Sun *et al.*, 2009)

Reaction	Method of immobilization
	Casting into a hydrophobically modified Nafion® membrane (Sokic-Lazic and Minteer, 2008)

viscosity that contribute a one-sided partition of high-molecular-weight macromolecules and evenly distributed low-molecular-weight molecules) and the use of multi-enzyme complexes that avoid diffusion problems, balancing stability and function (e.g., intolerance to impurities) against substrate specificity (Chakrabarti *et al.*, 2003; Bhattacharya *et al.*, 2004). However, in many ways, cells still hold the key to how their complex enzymatic cascades function so perfectly. Efforts currently being pursued in the "omics" sciences (genomics, transcriptomics, proteomics, metabolomics, etc.) will certainly unveil at least some of these details from which the design of future bioprocesses will benefit significantly. A better understanding of cell function and communication between enzyme molecules within metabolic networks will undoubtedly increase the productivity of known processes and pave the way to new enzymatic syntheses with coupled biocatalysts. Exciting developments in the use of multi-enzyme systems that rely on signaling crosstalk in enzyme-based biomolecular computing (using Boolean style logic) relies on a fascinating utilization of multi-enzyme signaling to process information, but the state of the art immobilizes enzymes individually (Pita *et al.*, 2008).

THE ARCHETYPE OF COOPERATING ENZYMES

Polyketide synthases (PKS), non-ribosomal peptide synthases and fatty acid synthases constitute the paradigm of sequentially acting enzymes. As in an industrial assembly line, substrates are handed from one functional domain to the next one (sometimes housed in the same polypeptide) to produce a wealth of structurally diverse molecules. Such molecules comprise metabolites and pharmaceutically important natural products including antibiotics, immunosuppressants, antiparasitics and anticancer compounds (Weissman and Leadlay, 2005; Weissman, 2008). The literature on the immobilization of these multi-enzymatic and multi-domain megasynthase enzymes, however, is scarce. To our knowledge the few reports on the immobilized use of such enzymes have been contributed by Dordick's research group (Srinivasan *et al.*, 2004; Ku *et al.*, 2006; Kwon *et al.*, 2007; Kim *et al.*, 2009). The use of a microfluidic reactor with immobilized Type III PKS (single iterative domains) coupled to a second immobilized peroxidase reactor, for example, produces a variety of flavonoids and pyrone derivatives (Kim *et al.*, 2009). However, the use of large modular multi-domain synthases (eg., Type I PKS) still remains a challenge. One of the reasons for the infrequent use of these valuable biocatalysts may lie in the difficulty of expression of such giant multi-enzymes and the hurdle of using such enzyme complexes *in vitro* (poor stability, low activity, difficulty to identify the products, etc.) (Staunton and Weissman, 2001; Betancor *et al.*, 2008). Future studies on the immobilization of megasynthases may set the basis for the formation of attractive molecules for a range of potential applications. The results would be not only of academic importance but also vital for more applied purposes such as the production of novel compounds by rational reconfiguring of these synthases or subtle modification of the enzyme structure by immobilization.

NEW AGE CARRIERS

The availability of new carriers for enzyme immobilization may be crucial for designing new processes involving sequentially acting enzymes. Material science is constantly providing us with new or improved supports. Recently, De Geest *et al* published the use

of self-exploding beads that release microcarriers (De Geest *et al.*, 2008). Upon variations in the pH of the medium and osmotic changes the outer layers of these polyelectrolyte-coated gel beads disintegrate, ejecting the inner microcapsules into the surrounding medium. Although the authors propose a possible and indeed useful application for the delivery of antigen-containing microcapsules within the body, the possibility to tailor the time of explosion could be of interest for the use of sequentially acting enzymes in certain applications (eg., when a certain amount of substrate is needed for the second enzyme to act or deleterious by-products need to be removed before the action of a sensitive subsequent enzyme).

Additionally, DNA scaffolds represent the ultimate example of new materials for enzyme immobilization with exciting future opportunities. Self-assembled single-stranded nucleic acids are able to form three-dimensional structures that can anchor DNA-tethered enzymes within the resulting scaffold. These structures have been proposed for the coimmobilization of enzyme cascades as they provide the possibility of controlling the reactivity of the system through the design of the individual DNA strips that form the scaffold (Wilner *et al.*, 2009).

ENZYME TECHNOLOGY IN *IN VITRO* SYSTEMS

As discussed, the advantages of using *in vitro* immobilized sequentially acting enzymes for bioprocesses are numerous. However, there are some problems inherent to the enzymes involved which still need to be overcome if we want the system to function repeatedly. Avoiding inactivation brought about by unfavourable reaction conditions could for instance greatly increase the economic feasibility of a process. In this regard, many scientific disciplines have contributed to provide an ever-increasing toolbox for the improvement of enzymatic properties. The design of tailor-made enzyme immobilization protocols, for example, has not only increased the stability of industrially relevant catalysts but also helps avoid inhibition problems and provide solutions for issues of enantioselectivity (Mateo *et al.*, 2007b). Protein aging can also be a significant reason for an enzyme-based system to cease functioning. The rate of some aging reactions can be reduced by carefully selecting proper reaction conditions such as oxygen content and pH. However, as the rate with which protein inactivation by covalent modifications proceeds is highly dependent on the specific sequence, the half-life of such proteins could potentially be improved by engineering the amino acid sequence (Hold and Panke, 2009). Similarly, the use of directed evolution has also been used to improve the activity recovery and stability of enzymes after immobilization to enhance biocatalysts (Ansorge-Schumacher *et al.*, 2006; Mateo *et al.*, 2007a). Moreover, site-directed mutagenesis of protein surfaces seems to be a powerful tool to greatly improve the immobilization and properties of the final immobilized biocatalyst and more effort may be expected in the next years in this regard. These are only a few examples of the many tools available to engineer putative immobilized enzymatic networks. Undoubtedly, the combined use of these tools will contribute to the rational design of integrated immobilized systems providing additional advantages over the use of engineered living systems.

Acknowledgements

Dr. Betancor gratefully recognizes funding from Ministerio di ciencia y technología (MCyT, Madrid; Ramón y Cajal research fellowship). Dr. Luckarift acknowledge-

ments research funding from the Air Force Office of Scientific Research (program manager, Walt Kozumbo) and the Air Force Research Laboratory, Materials Science Directorate, Biotechnology Program.

References

Ansorge-Schumacher, M. B., Slusarczyk, H., Schumers, J., Hirtz, D. (2006). Directed evolution of formate dehydrogenase from Candida boidinii for improved stability during entrapment in polyacrylamide. *FEBS Journal,* **273**, 3938-3945.

Averill, B. A., Laane, N. W. M., Straathof, A. J. J., Tramper, J. (1999). Biocatalysis. In *Catalysis: An integrated approach*, p. 346. Elsevier Science, Amsterdam, The Netherlands.

Beeckmans, S., Van Driessche, E., Kanarek, L. (1993). Immobilized enzymes as tools for the demonstration of metabolon formation. A short overview. *Journal of Molecular Recognition,* **6**(4), 195-204.

Berne, C., Betancor, L., Luckarift, H. R., Spain, J. C. (2006). Application of a microfluidic reactor for screening cancer prodrug activation using silica-immobilized nitrobenzene nitroreductase. *Biomacromolecules,* **7**(9), 2631-2636.

Betancor, L., Berne, C., Luckarift, H. R., Spain, J. C. (2006). Coimmobilization of a redox enzyme and a cofactor regeneration system. *Chemical communications,* (34), 3640-3642.

Betancor, L., Fernandez, M. J., Weissman, K. J., Leadlay, P. F. (2008). Improved catalytic activity of a purified multienzyme from a modular polyketide synthase after coexpression with Streptomyces chaperonins in *Escherichia coli*. *Chembiochem,* **9**(18), 2962-2966.

Betancor, L. AND Luckarift, H. R. (2008). Bioinspired enzyme encapsulation for biocatalysis. *Trends in Biotechnology,* **26**(10), 566-572.

Bhattacharya, S., Schiavone, M., Gomes, J., Bhattacharya, S. K. (2004). Cascade of bioreactors in series for conversion of 3-phospho-D-glycerate into D-ribulose-1,5-bisphosphate: kinetic parameters of enzymes and operation variables. *Journal of Biotechnology,* **111**(2), 203-217.

Bruggink, A., Schoevaart, R., Kieboom, T. (2003). Concepts of nature in organic synthesis: Cascade catalysis and multistep conversions in concert. *Organic Process Research & Development,* **7**(5), 622-640.

Chakrabarti, S., Bhattacharya, S., Bhattacharya, S. K. (2003). Biochemical engineering: cues from cells. *Trends in Biotechnology,* **21**(5), 204-209.

De Geest, B. G., McShane, M. J., Demeester, J., De Smedt, S. C., Hennink, W. E. (2008). Microcapsules ejecting nanosized species into the environment. *Journal of the American Chemical Society,* **130**(44), 14480-14482.

El–Zahab, B., Jia, H., Wang, P. (2004). Enabling multienzyme biocatalysis using nanoporous materials. *Biotechnology and Bioengineering,* **87**(2), 178-183.

Erhardt, F. A., Kugler, J., Chakravarthula, R. R., Jordening, H.-J. (2000). Co-immobilization of dextransucrase and dextranase for the facilitated synthesis of isomalto-oligosaccharides: Preparation, characterization and modeling. *Biotechnology and Bioengineering,* **100**, 673-683.

Filice, M., Vanna, R., Terreni, M., Guisan, J. M., Palomo, J. M. (2009). Lipase-catalyzed regioselective one-step synthesis of penta-*O*-acetyl-3-hydroxylactal. *European Journal of Organic Chemistry,* **2009**(20): 3327-3329.

Gamella, M., Campuzano, S., Reviejo, A. J., Pingarron, J. M. (2008). Integrated multienzyme electrochemical biosensors for the determination of glycerol in wines. *Analytica Chimica Acta,* **609**(2), 201-209.

Ghica, M. E., Pauliukaite, R., Marchand, N., Devic, E., Brett, C. M. (2007). An improved biosensor for acetaldehyde determination using a bienzymatic strategy at poly(neutral red) modified carbon film electrodes. *Analytica Chimica Acta,* **591**(1), 80-86.

Girelli, A. M. AND Mattei, E. (2005). Application of immobilized enzyme reactor in on-line high performance liquid chromatography: A review. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* **819**(1), 3-16.

Grazú, V., Abian, O., Mateo, C., Batista-Viera, F., Fernández-Lafuente, R., Guisán, J. M. (2005). Stabilization of enzymes by multipoint immobilization of thiolated proteins on new epoxy-thiol supports. *Biotechnology and Bioengineering,* **90**(5), 597-605.

Hanefeld, U., Gardossi, L., Magner, E. (2009). Understanding enzyme immobilisation. *Chemical Society reviews,* **38**(2), 453-468.

Hold, C. AND Panke, S. (2009). Towards the engineering of in vitro systems. *Journal of the Royal Society Interface,* **6**(Supp 4), S507-S521.

Huang, X., Holden, H. M., Raushel, F. M. (2001). Channeling of substrates and intermediates in enzyme-catalyzed reactions. *Annual Review of Biochemistry,* **70**, 149-180.

Irazoqui, G., Giacomini, C., Batista-Viera, F., Brena, B. M. (2007). Hydrophilization of immobilized model enzymes suggests a widely applicable method for enhancing protein stability in polar organic co-solvents. *Journal of Molecular Catalysis B: Enzymatic,* **46**(1-4), 43-51.

Jeykumari, D. R. AND Narayanan, S. S. (2008). Fabrication of bienzyme nanobiocomposite electrode using functionalized carbon nanotubes for biosensing applications. *Biosensors and Bioelectronics,* **23**(11), 1686-1693.

Kim, J., Grate, J. W., Wang, P. (2008). Nanobiocatalysis and its potential applications. *Trends in Biotechnology,* **26**(11), 639-646.

Kim, M. I., Kwon, S. J., Dordick, J. S. (2009). In vitro precursor-directed synthesis of polyketide analogues with coenzyme A regeneration for the development of antiangiogenic agents. *Organic Letters,* **11**(17), 3806-3809.

Kreft, O., Prevot, M., Mohwald, H., Sukhorukov, G. B. (2007). Shell-in-shell microcapsules: A novel tool for integrated, spatially confined enzymatic reactions. *Angewandte Chemie; International Edition,* **46**(29), 5605-5608.

Ku, B., Cha, J., Srinivasan, A., Kwon, S. J., Jeong, J. C., Sherman, D. H., Dordick, J. S. (2006). Chip-based polyketide biosynthesis and functionalization. *Biotechnology Progress,* **22**(4), 1102-1107.

Kwon, S. J., Lee, M. Y., Ku, B., Sherman, D. H., Dordick, J. S. (2007). High-throughput, microarray-based synthesis of natural product analogues via in vitro metabolic pathway construction. *American Chemical Society, Chemical Biology,* **2**(6), 419-425.

Lee, M. Y., Srinivasan, A., Ku, B., Dordick, J. S. (2003). Multienzyme catalysis in microfluidic biochips. *Biotechnology and Bioengineering,* **83**(1), 20-28.

Liu, S. AND Sun, Y. (2007). Co-immobilization of glucose oxidase and hexokinase on

silicate hybrid sol-gel membrane for glucose and ATP detections. *Biosensors and Bioelectronics,* **22**, 905-911.

LOGAN, T. C., CLARK, D. S., STACHOWIAK, T. B., SVEC, F., FRECHET, J. M. (2007). Photopatterning enzymes on polymer monoliths in microfluidic devices for steady-state kinetic analysis and spatially separated multi-enzyme reactions. *Analytical Chemistry,* **79**(17), 6592-6598.

LOPEZ–GALLEGO, F., BETANCOR, L., HIDALGO, A., MATEO, C., FERNANDEZ–LAFUENTE, R., GUISAN, J. M. (2005). One-pot conversion of cephalosporin C to 7-aminocephalosporanic acid in the absence of hydrogen peroxide. *Advanced Synthesis & Catalysis,* **347**(14), 1804-1810.

LUCKARIFT, H. R., KU, B. S., DORDICK, J. S., SPAIN, J. C. (2007). Silica-immobilized enzymes for multi-step synthesis in microfluidic devices. *Biotechnology and Bioengineering,* **98**(3), 701-705.

LUCKARIFT, H. R. (2008). Silica-immobilized enzyme reactors. *Journal of Liquid Chromatography and Related Technologies,* **31**(11-12), 1568-1592.

MAJER-BARANYI, K., ADANYI, N., VARADI, M. (2008). Investigation of a multienzyme based amperometric bisensor for determination of sucrose in fruit juices. *European Food Research Technology,* **228**, 139-144.

MATEO, C., GRAZÚ, V., PESSELA, B. C. C., MONTES, T., PALOMO, J. M., TORRES, R., LOPEZ-GALLEGO, F., FERNANDEZ-LAFUENTE, R., GUISAN, J. M. (2007a). Advances in the design of new epoxy supports for enzyme immobilization-stabilization. *Biochemical Society Transactions,* **35**, 1593-1601.

MATEO, C., PALOMO, J. M., FERNANDEZ-LORENTE, G., GUISAN, J. M., FERNANDEZ-LAFUENTE, R. (2007b). Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology,* **40**(6), 1451-1463.

MONTES, T., GRAZÚ, V., MANSO, I., GALÁN, B., LÓPEZ-GALLEGO, F., GONZÁLEZ, R., HERMOSO, J. A., GARCÍA, J. L., GUISÁN, J. M., FERNÁNDEZ-LAFUENTE, R. (2007). Improved stabilization of genetically modified penicillin G acylase in the presence of organic cosolvents by co-immobilization of the enzyme with polyethyleneimine. *Advanced Synthesis and Catalysis,* **349**(3), 459-464.

MULLER, J. AND NIEMEYER, C. M. (2008). DNA-directed assembly of artificial multienzyme complexes. *Biochemical and Biophysical Research Communications,* **377**(1), 62-67.

NAHALKA, J., LIU, Z., CHEN, X., WANG, P. G. (2003). Superbeads: immobilization in "sweet" chemistry. *Chemistry,* **9**(2), 372-377.

NIEMEYER, C. M., KOEHLER, J., WUERDEMANN, C. (2002). DNA-directed assembly of bienzymic complexes from in vivo biotinylated NAD(P)H:FMN oxidoreductase and luciferase. *Chembiochem,* **3**(2-3), 242-245.

NOUAIMI-BACHMANN, M., SKILEWITSCH, O., SENHAJI-DACHTLER, S., BISSWANGER, H. (2007). Co-immobilization of different enzyme activities to non-woven polyester surfaces. *Biotechnology and Bioengineering,* **96**(4), 623-630.

PITA, M., KRAMER, M., ZHOU, J., POGHOSSIAN, A., SCHONING, M. J., FERNANDEZ, V. M., KATZ, E. (2008). Optoelectronic properties of nanostructured ensembles controlled by biomolecular logic systems. *American Chemical Society, Nano,* **2**(10), 2160-2166.

QI, W., YAN, X., DUAN, L., CUI, Y., YANG, Y., LI, J. (2009). Glucose-sensitive microcapsules from glutaraldehyde cross-linked hemoglobin and glucose oxidase.

Biomacromolecules, **10**, 1212-1216.

ROMISCH, W., EISENREICH, W., RICHTER, G., BACHER, A. (2002). Rapid one-pot synthesis of riboflavin isotopomers. *Journal of Organic Chemistry,* **67**(25), 8890-8894.

SALINAS–CASTILLO, A., PASTOR, I., MALLAVIA, R., MATEO, C. R. (2008). Immobilization of a trienzymatic system in a sol–gel matrix: A new fluorescent biosensor for xanthine. *Biosensors and Bioelectronics,* **24**(4), 1053-1056.

SCHOEVAART, R. AND KIEBOOM, T. (2001). Combined catalytic reactions—Nature's way. *Chemical Innovation,* **31**(12), 33-39.

SCHOEVAART, R., VAN RANTWIJK, F., SHELDON, R. A. (2001). A four-step enzymatic cascade for the one-pot synthesis of non-natural carbohydrates from glycerol. *Journal of Organic Chemistry,* **66**(1), 351.

SHELDON, R. A. (2007). Enzyme immobilization: The quest for optimum performance. *Advanced Synthesis and Catalysis,* **349**(8-9), 1289-1307.

SOKIC-LAZIC, D. AND MINTEER, S. D. (2008). Citric acid cycle biomimic on a carbon electrode. *Biosensors and Bioelectronics,* **24**(4), 945-950.

SRINIVASAN, A., BACH, H., SHERMAN, D. H., DORDICK, J. S. (2004). Bacterial P450-catalyzed polyketide hydroxylation on a microfluidic platform. *Biotechnology and Bioengeering,* **88**(4), 528-535.

STAUNTON, J. AND WEISSMAN, K. J. (2001). Polyketide biosynthesis: a millennium review. *Natural Product Reports,* **18**(4), 380-416.

SUN, Q., JIANG, Y., JIANG, Z., ZHANG, L., SUN, X., LI, J. (2009). Green and efficient conversion of CO_2 to methanol by biomimetic coimmobilization of three dehydrogenases in protamine-templated titania. *Industrial & Engineering Chemistry Research,* **48**(9), 4210-4215.

VAMVAKAKI, V., HATZIMARINAKI, M., CHANIOTAKIS, N. (2008). Biomimetically synthesized silica-carbon nanofiber architectures for the development of highly stable electrochemical biosensor systems. *Analytical Chemistry,* **80**(15), 5970-5975.

VAN DE VELDE, F., LOURENCO, N. D., BAKKER, M., VAN RANTWIJK, F., SHELDON, R. A. (2000). Improved operational stability of peroxidases by coimmobilization with glucose oxidase. *Biotechnology and Bioengineering,* **69**(3), 286-291.

VAN DONGEN, S. F. M., NALLANI, M., CORNELISSEN, J. J. L. M., NOLTE, R. J. M., VAN HEST, J. C. M. (2009). A three-enzyme cascade reaction through positional assembly of enzymes in a polymersome nanoreactor. *Chemistry-A European Journal,* **15**, 1107-1114.

WANG, J., CHEN, L., HOCEVAR, S. B., OGOREVC, B. (2000). One-step electropolymeric co-immobilization of glucose oxidase and heparin for amperometric biosensing of glucose. *The Analyst,* **125**(8), 1431-1434.

WEISSMAN, K. J. AND LEADLAY, P. F. (2005). Combinatorial biosynthesis of reduced polyketides. *Nature Reviews: Microbiology,* **3**(12), 925-936.

WEISSMAN, K. J. (2008). Taking a closer look at fatty acid biosynthesis. *ChemBioChem,* **9**(18), 2929-2931.

WILNER, O. I., WEIZMANN, Y., GILL, R., LIOUBASHEVSKI, O., FREEMAN, R., WILNER, I. (2009). Enzyme cascades activated on topologically programmed DNA scaffolds. *Nature Nanotechnology,* **4**(4), 249-254.

ZAKS, A. (2001). Industrial biocatalysis. *Current Opinion in Chemical Biology,* **5**(2), 130-136.

ZHU, L., YANG, R., ZHAI, J., TIAN, C. (2007). Bienzymatic glucose biosensor based on co-

immobilization of peroxidase and glucose oxidase on a carbon nanotube electrode. *Biosensors and Bioelectronics*, **23**, 528-535.

Biotechnology & Genetic Engineering Reviews
Volume 27

Edited by Stephen E. Harding

Contents:

ISBN 978 1907 284557

Nottingham